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Soft tissue injuries and burn wounds constitute the greatest types of battlefield injuries. The goal of our research was to develop agents that would enhance healing of soft tissue injuries. The first objective was to establish the profile of growth factors and proteases during acute healing of skin wounds and to compare this with the profile of these agents in chronic wounds to establish what biochemical abnormalities need to be corrected to accelerate healing of slowly healing wounds. Our second objective was to evaluate the effect of topical treatment of exogenous growth factors on healing of autograph skin grafts in a pig model of full-thickness burns treated with partial thickness skin grafts. Our results demonstrate that cytokines, growth factors, their receptors and proteases play key roles in regulating healing of acute wounds. Furthermore, an imbalance of these agents which results in elevated levels of MMPs contributes to the impaired healing of chronic wounds. This implies that optimum treatment of battlefield injuries that are extensive including large burns or injuries with large tissue losses may require combination therapies that include growth factors and inhibitors of MMPs to achieve rapid healing. Topical treatment of pig partial thickness skin grafts with EGF accelerated healing indicating a potential treatment to accelerate healing of burn wounds.

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Gregory L. Brown, M. D.

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INTRODUCTION

Soft tissue injuries and burn wounds constitute the greatest types of battlefield injuries. The goal of our research was to develop agents that would enhance healing of soft tissue injuries. The first objective was to establish the profile of growth factors and proteases during acute healing of skin wounds and to compare this with the profile of these agents in chronic wounds to establish what biochemical abnormalities need to be corrected to accelerate healing of slowly healing wounds. Our second objective was to evaluate the effect of topical treatment of exogenous growth factors on healing of autograph skin grafts in a pig model of full-thickness burns treated with partial thickness skin grafts.

Wound healing in the skin is a complex biological process that has been extensively characterized at the light microscope level. However, regulation of skin wound healing is only partially understood at the molecular level. Skin wound healing can be divided into three general phases: (1) the inflammatory phase (2) the repair phase (3) and the remodeling phase. There is considerable temporal overlap of these stages of healing and the entire process lasts for several months [1,2].

The process of wound healing begins when the skin is injured intentionally (e.g., a surgical incision) or unintentionally (accidental trauma). Injury to blood vessels initiates blood clotting and platelet degranulation (Figure 1). Contained within the alpha granules of platelets are several growth factors including platelet-derived growth factor (PDGF) [3], insulin-like growth factor-I (IGF-I) [4], epidermal growth factor (EGF)[5], and transforming growth factor-beta (TGF-β)[6] . The burst of growth factors released from platelets quickly diffuses from the wound into the surrounding tissue and blood system. TGF-β released from platelets chemotactically recruits inflammatory cells into the injured area which initiates the inflammatory phase which peaks during the first 2 to 3 days. Neutrophils are the first major inflammatory cell to enter the wound. Neutrophils in the wound secrete more pro-inflammatory cytokines, engulf and destroy bacteria, and release proteases including the matrix metalloproteinases (MMPs), elastase and collagenase [7,9] that remove damaged extracellular matrix components. This early burst of protease activity in the wound is important in debriding the wound and is distinct from the later release of MMPs from fibroblasts during the remodeling phase of healing. Monocytes are chemotactically drawn into the wound by TGF-β or fragments of fibronectin about a day later than neutrophils and become activated macrophages [10]. Macrophages also secrete pro-inflammatory cytokines including tumor necrosis factor alpha (TNFα) and interleukin-1 (IL-1) and engulf and destroy bacteria. Macrophages also synthesize and secrete additional growth factors including TGF-β, transforming growth factor alpha (TGF-α), leukocyte derived growth factor (LDGF, a PDGF-like protein), basic fibroblast growth factor (bFGF), and heparin-binding epidermal growth factor (HB-EGF). If the wound does not become infected and is not subjected to repeated trauma or ischemia, the neutrophils disappear probably by undergoing apoptosis, and the inflammatory phase begins to decline. The growth factors secreted by macrophages continue to stimulate migration of fibroblasts, epithelial cells, and vascular endothelial cells into the wound setting up the repair phase of wound healing.

As the fibroblasts and vascular endothelial cells migrate into the site of injury, they begin to proliferate and the cellularity of the wound increases. The repair phase often lasts several weeks. As the number of macrophages in the wound begins to decrease, other cells in the wound such as fibroblasts, endothelial cells and keratinocytes continue to synthesize growth factors. Fibroblasts secrete IGF-1, bFGF, TGF-β, PDGF, and keratinocyte growth factor (KGF). Endothelial cells produce bFGF and PDGF. Keratinocytes synthesize TGF-β and TGF-α. These growth factors continue to stimulate proliferation, synthesis of extracellular matrix proteins, and angiogenesis.

After the initial scar forms, proliferation and neovascularization cease, and the wound enters the remodeling phase which can last for many months. During this last phase, a balance is reached

between the synthesis of new components of the scar matrix and their degradation by MMPs such as collagenase, gelatinase, and stromelysin. Fibroblasts are the major cell type responsible for synthesis of the extracellular matrix components collagen, elastin and proteoglycans. Fibroblasts also are an important source of the MMPs that degrade the matrix. They also secrete the tissue inhibitors of metalloproteinases (TIMPs) and lysyl oxidase which cross links components of the extracellular matrix. In later stages, angiogenesis ceases and capillary density in the wound site decreases. Eventually the scar tissue reaches equilibrium between deposition and removal of matrix although the mature scar is never as strong as uninjured skin.

Burn wounds or wounds caused by exposure to chemical agents such as acid or base have the additional problem of denatured ECM components. Skin grafting is frequently the only treatment that is effective in healing these wounds. In our previous studies supported by the US Army, we investigated the effects of EGF on cells in culture, in animal models of skin wound healing and in a clinical of partial thickness wound healing. These studies were very successful and were the basis for the current experiments.

The ability of EGF and TGF-α to stimulate processes *in vitro* that are important in wound healing such as migration, mitosis, angiogenesis and ECM deposition suggested that exogenously applied EGF or TGF-α might enhance healing of wounds *in vivo*. EGF and other members of the EGF family were first evaluated for their effects on enhancing healing of wounds in animal models. Topical application of EGF accelerated the rate of epidermal regeneration of partial thickness burns or dermatome wounds created on pigs [11,14]. EGF was also reported to increase tensile strength of surgical skin incisions during the early period of healing in normal rats [15] and to increase formation of granulation tissue in subcutaneous sponges in rats when applied in a slow release vehicle [16]. EGF also increased deposition of collagen in cylinders implanted subcutaneously in rats treated with streptozotocin to impair healing [17].

In the first clinical trial of a peptide growth factor, recombinant human EGF accelerated epidermal regeneration of paired dermatome wounds in patients requiring skin grafting [18]. Paired donor sites were created in 12 patients who required skin grafting for burns or reconstructive surgery. One donor site of each patient was treated daily with vehicle (silver sulfadiazine cream) containing recombinant human EGF (10 μg/ml) while the other donor site was treated with the vehicle. Treatment with EGF significantly decreased the average length of time to 50% healing by 1 day and decreased the average time to complete healing by 1.5 days ($p=0.02$). These experiments suggested that growth factor treatment could enhance healing of skin wounds and emphasized the need to biochemically characterize healing and impaired wounds to identify the approaches that would potentially stimulate healing of acute and impaired wounds.

The methods utilized to biochemically characterize the molecular environment of acute and chronic wounds were to collect fluids and biopsies from typical healing and non-healing skin wounds then use bioassays, RIAs, immunohistochemistry and quantitative reverse transcription polymerase chain reaction (Q-RT-PCR) to measure the levels of mRNAs and proteins.

RESULTS OF EXPERIMENTS

Our concept of normal wound healing is based on the model that wound healing is regulated by the sequential, balanced action of cytokines, proteases and growth factors. A corollary to this concept is that elevated levels of cytokines induce excessive levels of proteases which impair wound healing by degrading ECM proteins, growth factors and their receptors. If this concept is true, then therapies that re-establish an environment in chronic wounds that permit growth factors to function normally should lead to healing of chronic wounds. To investigate this hypothesis, we collected fluids from human wounds that represent the extreme ends of the spectrum of wound healing: acute, healing mastectomy incisions and chronic, nonhealing skin ulcers. A model of our

hypothesis is shown in Figure 1.

Cytokine Levels in Wound Environments

Cytokine levels in acute human wound fluids. Our initial analysis of cytokines in 21 samples of acute wound fluid collected from 5 patients during their post-surgery period and 5 samples of chronic wound fluid are shown in Figure 2, panels A through F. TNFα levels peaked on days 2 and 4 after surgery (50 pg/ml) and returned to near baseline values on days 5, 6, and 7 after surgery. Levels of IL-1β (25 pg/ml) and IL-6 (35 pg/ml) were highest on day 1 after surgery and progressively decreased to baseline levels from days 2 to 7. Levels of IL-8 (5,000 pg/ml) and INFγ (4 pg/ml) were relatively constant across the time from days 1 to 8 after surgery.

In addition to regulation at the level of transcription, the activities of TNFα and IL-1β can be modulated post-translationally by naturally occurring proteins which can bind to the cytokine or its receptor. A soluble form of the TNFα receptor protein, p55, binds TNFα much like a neutralizing antibody and inhibits TNFα activity. Levels of p55 were approximately 300 pg/ml in acute wound fluid samples collected during the 8 days following mastectomy. Thus, the ratio of p55/TNFα in acute wound fluids is about 6 to 1 in favor of p55 to TNFα (300 pg/ml verses 50 pg/ml). Levels of IL-1ra, the natural inhibitor of IL-1α and IL-1β, were approximately 8,000 pg/ml in the 21 acute wound fluid samples. The ratio of IL-1ra to IL-1β in acute wound fluids was about 320 to 1 in favor of the IL-1ra (8,000 pg/ml verses 25 pg/ml). In summary, the ratios of antagonist/inflammatory cytokines for TNFα and IL-1β in the molecular environment of acute wounds strongly favor the inhibitors indicating the effects of TNFα and IL-1β are finely regulated.

Cytokine levels in chronic human wound fluids. In chronic wound fluids, the levels of TNFα and IL-1β were higher and more variable than in acute wound fluids (Figure 2). TNFα levels averaged 500 pg/ml which was 10-fold higher than in acute wound fluids. IL-1β levels averaged 2,500 pg/ml which was about 100-fold higher than levels in acute wound fluids. Levels of INFγ were about 3-fold higher in chronic wounds than acute wounds (4 vs 15 pg/ml). Levels of IL-6 (30 pg/ml) were 2- to 4-fold higher in chronic wounds than the levels measured in late acute wound fluids. Levels of IL-8 were low and similar in chronic and acute wound fluids (about 5,000 pg/ml). While the levels of p55 in chronic wound fluids were substantially higher than in acute wound fluids (approximately 1,700 pg/ml vs 300 pg/ml), the ratio of p55/TNFα in chronic wound fluids had decreased 2-fold to about 3 to 1 in favor of p55 (1,700 pg/ml verses 500 pg/ml). In contrast, the average level of IL-1ra in chronic wound fluids was lower than in acute wound fluids (approximately 3,000 pg/ml vs 8,000 pg/ml). The decrease in IL-1ra caused the ratio of IL-1ra to IL-1β in chronic wound fluids to decrease from 320 to 1 in acute wounds to about an approximately equal ratio of 1.2 to 1 in chronic wounds. In summary, these data indicate that the biological effects of TNFα, IL-1β, INFγ, and IL-6 should be much greater in the environment of chronic wounds than in acute wounds.

Protease and Inhibitor Levels

The second component of our hypothesis of the molecular pathophysiology of chronic wounds is that elevated levels of TNFα and IL-1β synergistically increase levels of MMPs and decrease levels of TIMPs in chronic wounds. The elevated protease activities degrade components in the wound environment that are essential for healing including extracellular matrix proteins, growth factors and their receptors. In addition, the fragments of ECM components, such as fibronectin, generated by the proteases act as chemotactic factors for inflammatory cells. This further increases the number of inflammatory cells drawn into the wound and creates a self-amplifying cycle that elevates protease levels. We have analyzed a substantial number of fluids

from acute and chronic human wounds for several proteases and inhibitors using a number of different assays. These include gelatin zymography, casein zymography, Azocoll proteolysis assay, Azocasein proteolysis assay, growth factor degradation assay, neutrophil elastase assay, neutrophil elastase inhibitor assay, and cathepsin G assay. The results of these assays consistently demonstrate elevated levels of proteases and reduced levels of inhibitors in chronic wound fluids compared to acute wound fluids.

Azocoll Assay of Acute, Chronic and Sequential Wound Fluids Our initial data indicates there are major differences in the levels of protease activity between acute and chronic wound fluids. The average level of protease activity in 20 different acute wound fluids collected from mastectomy incisions, determined using Azocoll as the substrate [19], is low (0.75 µg collagenase equivalents/ml), with a range of 0.1 to 1.3 µg collagenase equivalents/ml. Also, the average levels do not change substantially during the first 7 days after surgery. In addition, the levels of TIMP-1 determined using an ELISA in three acute wound fluids is 38 ± 6 µg/ml (Figure 3A). The ELISA detects both free TIMP-1 and TIMP-1/MMP complexes although not with the same efficiency as free TIMP-1. This suggests that protease activity is tightly controlled during the early phase of wound healing.

In contrast, the range of Azocoll protease activity in chronic wound fluids collected before clinical treatments had begun is quite large, ranging from 5 to 584 µg collagenase equivalents/ml. The average level of protease activity in 32 different chronic wound fluids was 87 ± 24 µg collagenase equivalents/ml which is 116-fold higher ($p<0.05$) than in mastectomy fluids. Furthermore, addition of the MMP inhibitor, Galardin, to chronic wound fluids reduced hydrolysis of Azocoll by an average of 90%. This indicates that the majority of proteases in these chronic wound fluids which degrade Azocoll are MMPs (Figure 3B). In addition, the levels of TIMP-1 measured in 5 of these chronic wounds was inversely related to the level of Azocoll hydrolysis activity. As shown in Figure 3A, samples CW-33 and CW-25 had low levels of Azocoll hydrolysis and had high levels of TIMP-1. In contrast, chronic wound fluids I-24, I-29, and I-42 had low levels of TIMP-1 that were 50 to 100-fold lower than levels in acute wound fluids which had low levels of Azocoll hydrolysis. Similar results were found for neutrophil elastase and its inhibitor and cathepsin G.

We also analyzed an initial series of sequential wound fluids collected from 10 patients with venous stasis ulcers before beginning treatments and collected again after two weeks of treatment when the ulcers typically are about 30% healed. Our hypothesis predicts that the levels of MMPs should decrease in chronic wound fluids as healing progresses. To standardize the collection process, these patients were hospitalized the night before beginning treatment and had no fluids after midnight. At 8 AM the next morning, they drank 1 liter of water, the ulcer was covered with occlusive dressing and the ulcer was placed in a dependent position. After 1 hour the fluid that had spontaneously collected was removed and analyzed.

The average level of Azocoll hydrolysis was quite high in the 10 samples collected before therapy began (52 ± 12 µg collagenase equivalents/ml). After two weeks of treatment, the levels of Azocoll hydrolysis decreased in 8 of 10 of the patients to an average level of 15 ± 6 µg collagenase/ml. Overall, this is a 3.4-fold decrease in protease levels of the 10 patients. We also assessed the effects of addition of the MMP inhibitor, Galardin, on the Azocoll hydrolysis by the sequential wound fluids. Galardin inhibited almost all the Azocoll hydrolysis activity in the sequential wound fluids. For example, Galardin decreased the average Azocoll hydrolysis activity by 99.2% from 52 µg collagenase/ml to 0.4 µg/ml in the samples collected before treatment began. Galardin also effectively decreased protease activity in fluids collected two weeks after treatment began from 15 µg collagenase/ml to 0.4 µg/ml.

Gelatin and Casein Zymography of Acute, Chronic, and Sequential Wound Fluids Results of the Azocoll assay and Galardin inhibition indicated that the wound fluids contained activated

MMPs. To help identify which MMPs might be present and activated, we performed gelatin and casein zymograms of acute and chronic wound fluids [20]. As shown in Figure 4, analysis of three acute wound fluids collected on postoperative days 1, 2, and 3 revealed the presence of one major band of molecular weight 92 kDa and two minor bands at molecular weights of approximately 130 kDa and 200 kDa. Based on migration of purified samples of MMPs, we identified these bands as the pro-MMP-9 (92 kDa gelatinase, gelatinase B) and complexes of MMP-9 with other proteins. The activated forms of MMP-9 and MMP-2 migrated as slightly lower molecular weight bands. There was an extremely faint band migrating at 89 kDa in acute fluid POD-1 which is probably activated MMP-9 which is consistent with the results of the Azocoll assay that showed very low levels of protease activity.

The gelatin zymogram of five chronic wound samples showed dramatically different patterns of bands from the acute wound fluids. The samples 1 through 5, Figure 4 had a large range of protease activity determined by the Azocoll assay of 3, 3.5, 22, 584 and 130 µg/ml, respectively. The band pattern for sample 2 was very similar to the acute wound fluid with three major bands, but the higher molecular weight bands were more intense and there was a faint diffuse band at a molecular weight range of 35 to 55 kDa. Chronic wound sample 1 had an intense band at 85 kDa and the diffuse band at 35 to 55 kDa. Samples 3, 4, and 5 all had multiple, intense bands especially at 85 kDa (activated MMP-9), 68 kDa (probably activated MMP-2) and 35 to 55 kDa (probably MMP-3). MMP-1, or collagenase, is not readily detected by gelatin zymography since it makes a single cut in native collagen. Thus, gelatin zymograms of acute and chronic wound fluids support the quantitative measurements of protease activity made with the Azocoll assay and indicate the presence of several active MMPs in chronic wound fluids.

We also analyzed the sequential wound fluids using gelatin and casein zymography. The patterns of the fluids collected from 10 patients with chronic venous stasis ulcers before they began conventional compression therapy had six, intense bands at approximate molecular weights of >200 kDa, 150 kDa, 100 kDa, 85 kDa, 65 kDa, and 45 kDa. These patterns were more intense in the sample collected from each patient before treatment began compared to the second sample collected after two weeks of treatment. This is particularly apparent in the pair of samples numbered 79 and 80. For example, the initial sample collected before treatment had very intense bands especially at the three higher molecular weights (>200 kDa, 150 kDa, 100 kDa) and faint bands at <45 kDa. In contrast, the bands generated by the second sample collected two weeks after treatment began were much less intense and no activated MMP-9 (92 kDa gelatinase B) was detected. Incubation of an identical gel in buffer containing Galardin during the development period totally blocked band formation indicating that all the bands detected by the gelatin zymogens were MMPs.

Q-RT-PCR Analysis of MMP and TIMP of Chronic Wound Biopsies Using competition-based quantitative RT-PCR [21], we also measured the levels of mRNAs for MMP-1, MMP-2, MMP-3, MMP-9, TIMP-1 and TIMP-2 in biopsies from normal skin, the edge of each ulcer and from the base of 8 different chronic wounds (Figure 5). The levels of mRNAs agreed well with the levels of proteins measured in chronic wound fluids. Specifically, levels of mRNAs in the biopsies from the base of the wounds were elevated 300-fold, 200-fold, 100-fold and 450-fold for MMP-1, MMP-2, MMP-3, MMP-9, respectively compared to normal skin. In contrast, the levels of mRNAs for TIMP-1 and TIMP-2 were only increased 30-fold and 15-fold in the wound tissue compared to normal skin. Levels of mRNAs at the edge of the wound tended to be about half as high as the levels in the base of the ulcers. Thus, the levels of mRNAs for MMPs were consistently elevated several hundred fold in chronic wounds compared to levels in normal skin whereas mRNAs for TIMPs were increased substantially less.

In summary, these data indicate that the environment of acute wounds have low levels of active MMPs and high levels of TIMP-1 proteins. In contrast, most chronic wound fluids contain

high levels of activated MMPs and low levels of TIMP-1. Levels of mRNAs measured by Q-RT-PCR agree with the trend of the protease measurements. The physiological implications of these data are that MMP activity is tightly controlled in acute wounds due to the presence of TIMPs (and to low levels of TNFα and IL-1β and elevated levels of p55 and IL-1ra). In chronic wounds, however, levels of active MMPs are elevated and levels of TIMPs are decreased (presumably induced by high levels of TNFα and IL-1β and by low levels of p55 and IL-1ra). These data support our hypothesis that the elevated inflammatory cytokines induce synthesis of MMPs in chronic wounds. In the next section, we examine what biological effects the elevated levels of proteases may have on the biological activities of essential ECM proteins, growth factors and receptors.

Growth Factor Degradation Our hypothesis of the molecular pathophysiology of chronic wounds predicts that elevated levels of proteases in chronic wound fluids destroy essential ECM, growth factors and their receptors which prevents wounds from healing. To assess if growth factors are stable or are destroyed in acute and chronic wound fluids, we added iodinated EGF, PDGF and IGF-I to samples of acute and chronic wound fluid and measured their stability by precipitation with trichloroacetic acid (TCA). These growth factors are insoluble in 15% TCA whereas small fragments generated by proteolysis are soluble in 15% TCA. As shown in Figure 6, the results were essentially the same as we found using Azocoll as the substrate. Only 2 of 20 mastectomy fluids caused any measurable destruction (0.05%) of the added EGF (0.15 ± 0.01 μg EGF degraded/ml wound fluid/24 hours) while all 14 different chronic wound fluids caused substantial destruction of the added EGF (average of 16.72 ± 6.07 μg EGF degraded/ml wound fluid/24 hours, 60% of the added EGF). Addition of Galardin or EDTA prevented the destruction of EGF to the low levels measured in acute wound fluids. Similar results were obtained for PDGF and IGF-I using samples of our acute and chronic wound. The different amounts of degradation of growth factors in acute and chronic wound fluids are not due to higher levels of total proteins in acute wound fluids simply acting as a "protease sink" and sparing the growth factors since the levels of total proteins in acute and chronic wound fluids are both approximately 6 to 8 grams per 100 ml. In addition, the pH and osmolarity of chronic wound fluids were not significantly different from normal serum: the average pH was 7.26 ± 0.16 n=7 and the average osmolarity was 312 ± 13 mosmoles, n=15).

Our hypothesis predicts that the levels of biologically active growth factors should be high in acute wound fluids while levels in chronic wound fluids should be low. Our initial analyses of growth factors in acute and chronic wound fluids support this general concept. Mastectomy fluids contained physiologically significant levels of four growth factors (TGF-α, EGF, TGF-β, and IGF-I) at concentrations which can stimulate mitosis and migration of cells in culture as well as induce or suppress transcription of genes *in vitro*. Second, the levels of EGF and IGF-I are lower in chronic wound fluids compared to acute wound fluids. However, average levels of TGF-α and TGF-β are slightly elevated in chronic wound fluids. This may be due to the detection of proteolytic fragments of TGF-α in wound fluids by the RIA.

Growth Factor Receptor Degradation Receptor proteins are another class of proteins which are necessary for growth factors and cytokines to function in wound healing. In an initial experiment investigating the stability of EGF receptors incubated with acute and chronic wound fluids, we found that the EGF receptor was indeed stable in acute wound fluids but not in chronic wound fluids. Preincubation of human placental microvilli membranes (a very rich source of EGF receptors) with two mastectomy fluids for 2 hours did not reduce binding of ^{125}I-EGF to receptors. In contrast, preincubation of the placental membranes with two chronic wound fluids reduced EGF binding by 40% and 60%. Addition of EDTA or Galardin prevented the destruction of EGF-R by chronic wound fluids indicating that MMPs were responsible for the inactivation of the EGF-R. Thus, proteases in chronic wound fluids can degrade EGF receptors and may impede wound healing *in vivo* by degrading both growth factors and their receptors.

Biological Effects of Acute and Chronic Wound Fluids on Wound Cells

The final stage of our hypothesis of chronic wounds predicts that fluids from acute wounds should stimulate essential processes of wound healing such as DNA synthesis of wound cells while chronic wound fluids would not. We evaluated the ability of a series of acute and chronic wound fluids to influence DNA synthesis of the three major types of wound cells: fibroblasts, keratinocytes, and vascular endothelial cells.

Addition of mastectomy fluids to serum-free chemically defined medium stimulated high levels of DNA synthesis by cultures of human foreskin fibroblasts. As seen in Figure 7A, mastectomy fluids collected during days 1 and 2 after surgery stimulated DNA synthesis to levels slightly higher than addition of 10% calf serum, while fluids collected during days 4, 5, and 7 after surgery stimulated levels slightly below 10% serum. In marked contrast, 13 of 14 fluids collected from chronic wounds decreased DNA synthesis of fibroblast cultures when added to serum-free medium (Figure 7B). Similar effects were found when acute and chronic wound fluids were added to cultures of human umbilical vein endothelial cells held in low serum (2%). Mastectomy fluids collected on days 1, 2, 3, and 5 after surgery increased DNA synthesis approximately 2-fold above low serum while 4 of 5 chronic wound samples substantially decreased DNA synthesis. Wound fluids produced similar effects on keratinocyte cultures. Acute wound fluids collected from 5 patients on days 1, 2, 3, 4, 5, and 7 supported DNA synthesis while fluids from 8 of 12 chronic wounds reduced DNA synthesis an average of 55%. Thus, fluids from acute wounds promote DNA synthesis while fluids from chronic wounds impair DNA synthesis.

Effects of Topical EGF on Healing of Large Meshed Graft Fenestrations

It is well known that meshed skin grafts extend the area that can feasibly be resurfaced for excised third degree burns. Therefore, the larger the meshed graft fenestrations the more excised burn that can be covered from a given donor area. We measured the rate of meshed graft fenestration (3:1) closure treated topically with an epidermal growth factor (EGF) containing solution compared to a placebo or normal saline solution.

Four Yorkshire mini-pigs had four 4 x 4 cm full-thickness wounds created on their thoracic paraspinous region. Four split-thickness skin grafts were harvested from the lumbar region. These grafts were meshed with a 3:1 carrier. The meshed grafts were stapled to the full-thickness wounds and secured in placed with fluffed gauze followed by a Reston stint.

Two grafts on each piglet were topically treated twice daily with normal saline (10 ml per dose) and two grafts were treated twice daily with normal saline containing EGF (10 micrograms/ml) in a double blinded study design.

The stints were removed at 4 days postoperatively for photographic analysis; the stints were reapplied and the treatment regimen resumed until the 7th postoperative day. The stints were once again removed and the wounds photographed.

The photographs were analyzed by planimetric analysis such that the area of each fenestration was determined for a given wound.

Results: percentage fenestration closure n=8

	EGF	Placebo	
Day 4	68%	42%	p=0.01
Day 7	94%	80%	p=0.05

We conclude from these experiments that topically applied EGF to a meshed graft can accelerate

fenestration closure.

CONCLUSIONS

Our results demonstrate that cytokines, growth factors, their receptors and proteases play key roles in regulating healing of acute wounds. Furthermore, an imbalance of these agents which results in elevated levels of MMPs contributes to the impaired healing of chronic wounds. This implies that optimum treatment of battlefield injuries that are extensive including large burns or injuries with large tissue losses may require combination therapies that include growth factors and inhibitors of MMPs to achieve rapid healing.

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APPENDIX

APPENDIX I

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1. N. Chegini, M.J. Rossi, G.S. Schultz, W.A. Dunn, Jr., and B.J. Masterson. Cellular Distribution of EGF, TGF-α and bEGF Receptor in Fascia Peritoneum During Healing: an Autoradiographic and Immunohistochemical Study. **Wound Rep Reg** 1:28-40, 1993.

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13. P. Malcherek, G. Schultz, U. Wingren, and L. Franzen. Formation of Healing Tissue and Angiogensis in Repair of Connective Tissue Stimulated by Epidermal Growth Factor. **Scan J Plast Reconstr Hand Surg** 28:1-7, 1994.

14. A.V. Nall, R.E. Brownlee, C.P. Colvin, G.S. Schultz, D. Fein, T Lui, A. Kalra, N.J. Cassisi. Transforming growt factor Beta-1 Improves Wound Healing and Random Flap Survival in Normal and Irradiated Rats. **Arch Otolaryngol Head Neck Surgery**, in press

15. R.W. Tarnuzzer, S.P. Macauley, W.G. Farmerie, S. Cabellaro, M.R. Ghassemifar, J.T. Anderson, C.R. Robinson, M.B. Grant, M.G. Humphreys-Beher, L. Franzen, A.B. Peck, and G.S. Schultz. Competitive RNA Templates for Detection and Quantification of Growth Factors, Cytokines, Extracellular Matrix Components, and Matrix Metalloproteinses by RT-PCR. **Biotechniques**, in press

APPENDIX II

FIGURE LEGENDS

Figure 1. Molecular pathophysiology of chronic wounds.

Figure 2. Cytokine levels in acute and chronic wound fluids. A. Concentrations of TNF alpha in mastectomy fluids; B. Concentrations of TNF alpha in chronic wound fluids; C. Concentrations of IL-1 beta in mastectomy fluids; D. Concentrations of IL-1 beta in chronic wound fluids; E. Concentrations of IL-6 in mastectomy fluids; F. Concentrations of IL-6 in chronic wound fluids. POD is postoperative day in mastectomy fluids and I- designation is for chronic wound fluids from various samples.

Figure 3. Determination of protease and inhibitor levels in acute wound fluids. A. Protease levels in mastectomy and chronic wound fluids were determined by Azocoll assay. TIMP-1 levels in wound fluids were determined by ELISA. B. Determination of protease levels in chronic wound fluids with Azocoll and inhibition by Galardin.

Figure 4. Gelatin zymography of wound fluids. Visualization of gelatinolytic proteinases in acute and chronic wound fluids.

Figure 5. Quantitative RT-PCR measurement of mRNA levels for MMPs and TIMPs.

Figure 6. Degradation of EGF by wound fluids. Radiolabeled EGF was incubated with samples of acute and chronic wound fluids and degradation determined by measurement of TCA precipitable counts.

Figure 7. Effects of wound fluids on DNA synthesis. A. Effect of mastectomy fluids on wound fibroblasts tritiated thymidine incorporation. B. Effect of chronic wound fluids on wound fibroblast tritiated thymidine incorporation.

Figure 1.

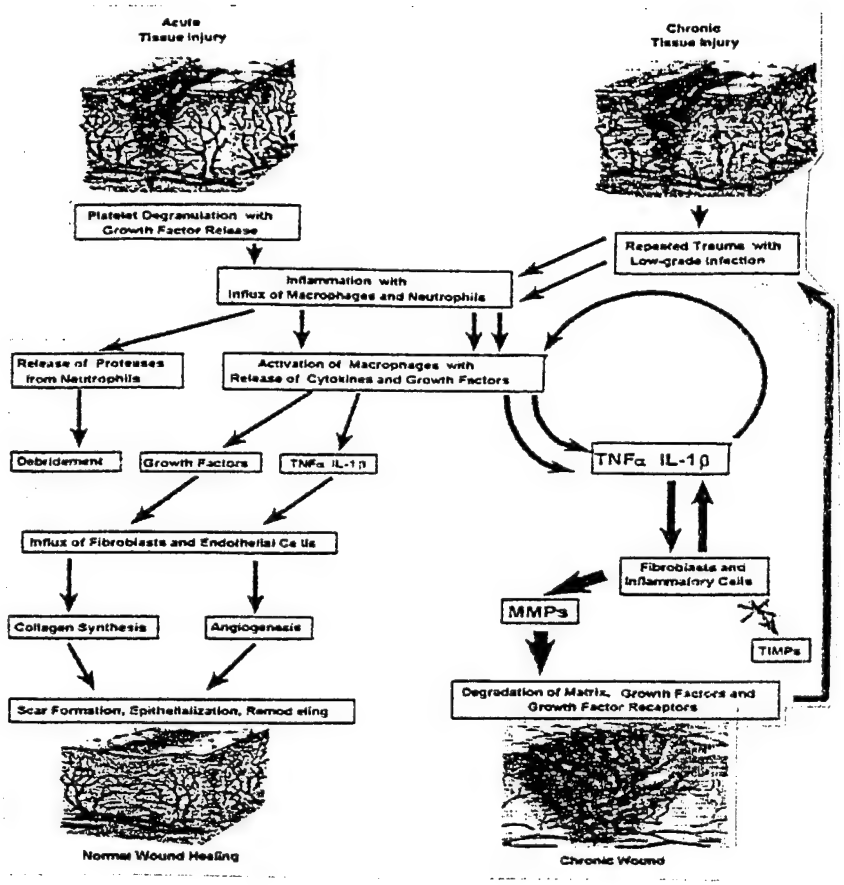

Acute
Tissue Injury
Chronic
Tissue Injury
Platelet Degranulation with
Growth Factor Release
Repeated Trauma with
Low-grade Infection
Inflammation with
Influx of Macrophages and Neutrophils
Release of Proteases
from Neutrophils
Activation of Macrophages with
Release of Cytokines and Growth Factors
Debridement
Growth Factors
TNFα IL-1β
TNFα IL-1β
Influx of Fibroblasts and Endothelial Cells
Fibroblasts and
Inflammatory Cells
MMPs
Collagen Synthesis
Angiogenesis
TIMPs
Scar Formation, Epithelialization, Remodeling
Degradation of Matrix, Growth Factors and
Growth Factor Receptors
Normal Wound Healing
Chronic Wound

Figure 2.

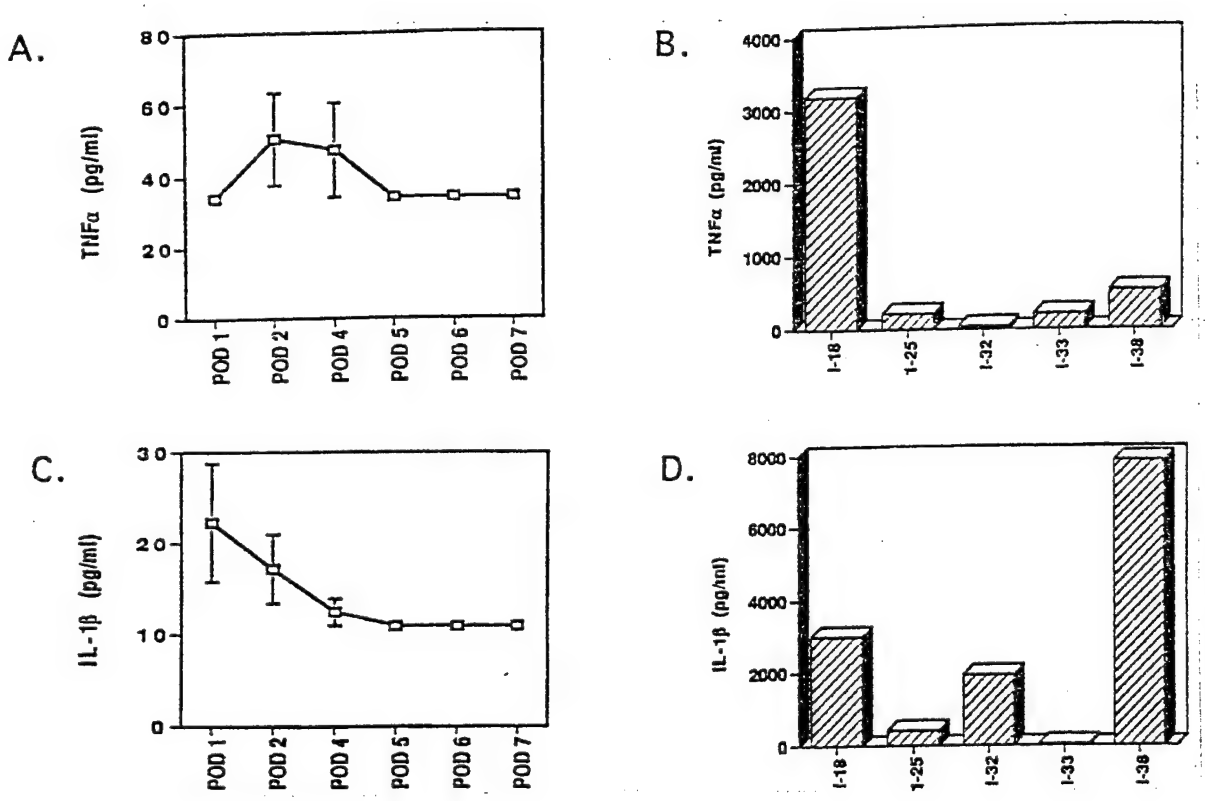

A.
TNFα (pg/ml)
80
60
40
20
0
POD 1
POD 2
POD 4
POD 5
POD 6
POD 7
B.
TNFα (pg/ml)
4000
3000
2000
1000
0
I-18
I-25
I-32
I-33
I-38
C.
IL-1β (pg/ml)
30
20
10
0
POD 1
POD 2
POD 4
POD 5
POD 6
POD 7
D.
IL-1β (pg/ml)
8000
6000
4000
2000
0
I-18
I-25
I-32
I-33
I-38

Figure 3.

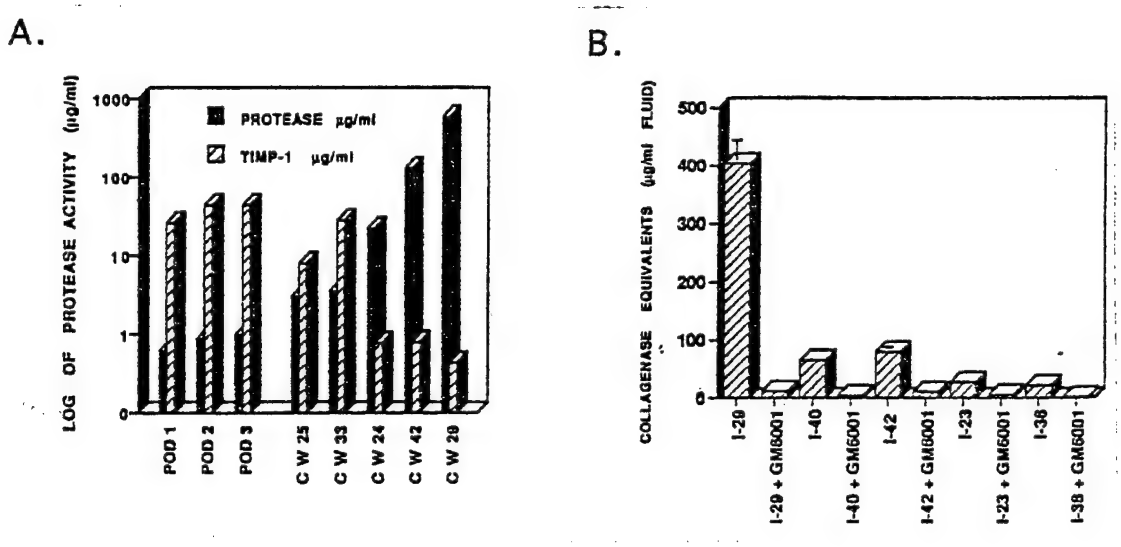

A.
LOG OF PROTEASE ACTIVITY (µg/ml)
1000
100
10
1
0
PROTEASE µg/ml
TIMP-1 µg/ml
POD 1
POD 2
POD 3
C W 25
C W 33
C W 24
C W 42
C W 29
B.
COLLAGENASE EQUIVALENTS (µg/ml FLUID)
500
400
300
200
100
0
I-29
I-29 + GM6001
I-40
I-40 + GM6001
I-42
I-42 + GM6001
I-23
I-23 + GM6001
I-38
I-38 + GM6001

Figure 4.

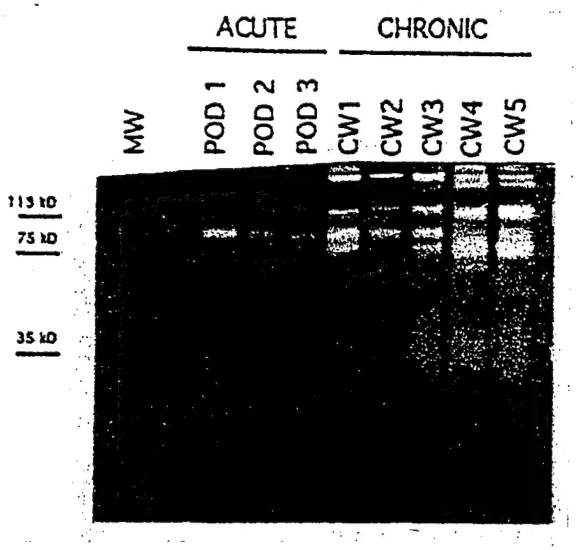

ACUTE
CHRONIC
MW
POD 1
POD 2
POD 3
CW1
CW2
CW3
CW4
CW5
113 kD
75 kD
35 kD

Figure 5.

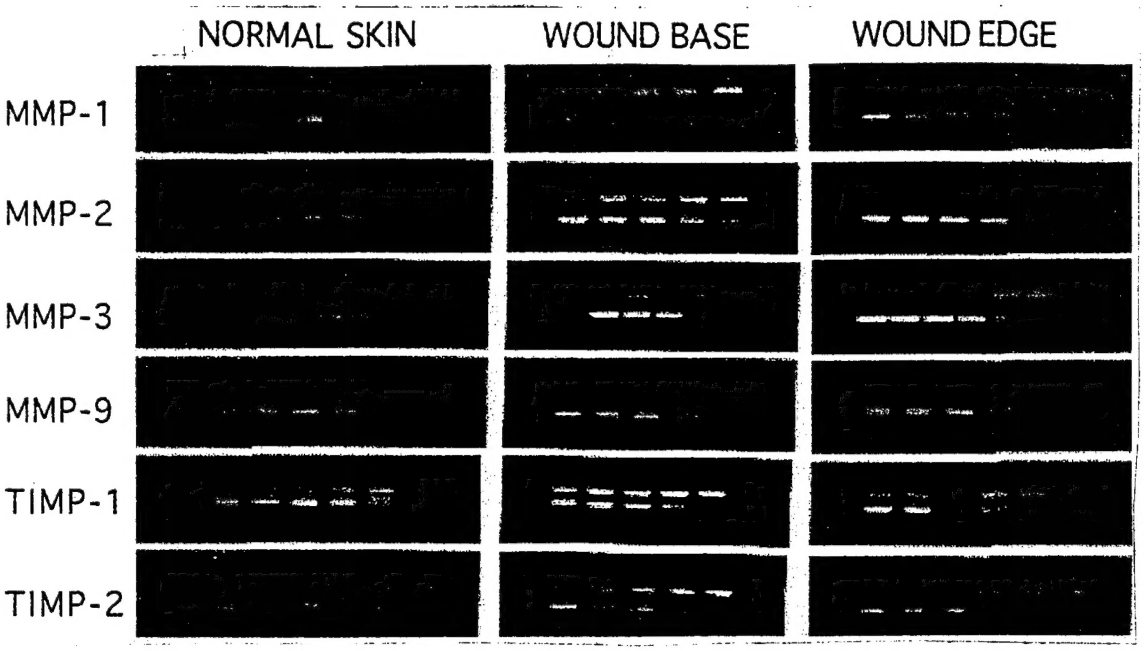

NORMAL SKIN
WOUND BASE
WOUND EDGE
MMP-1
MMP-2
MMP-3
MMP-9
TIMP-1
TIMP-2

Figure 6.

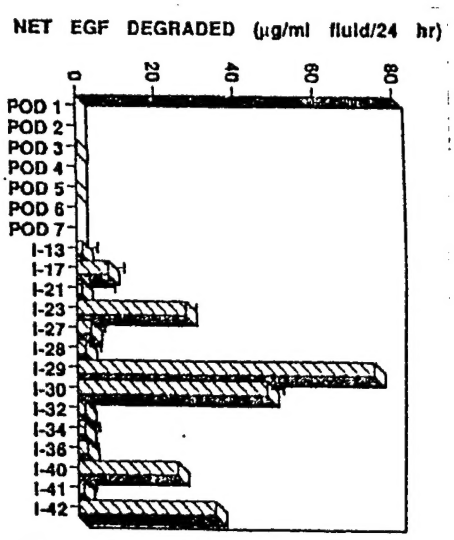
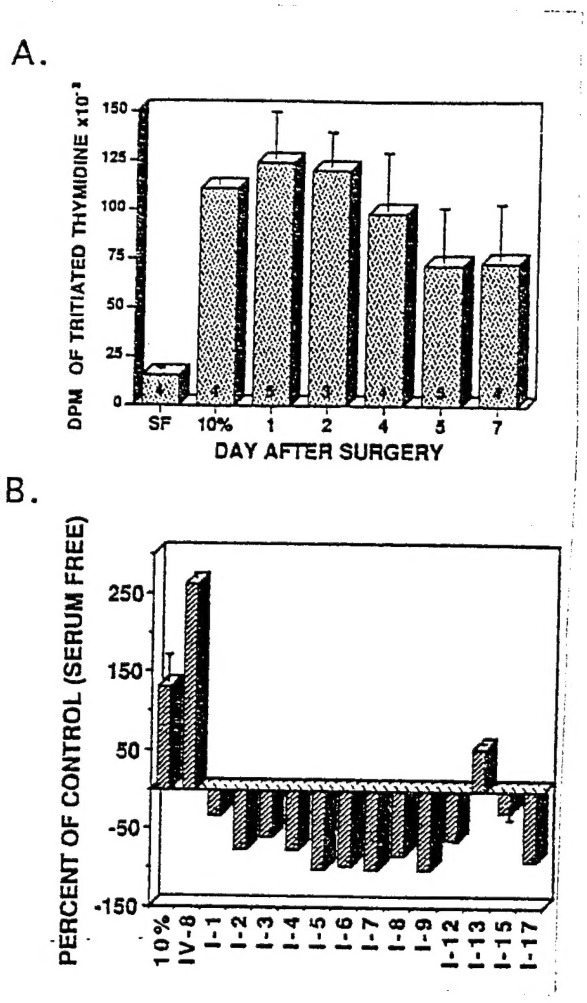

NET EGF DEGRADED (µg/ml fluid/24 hr)
0
20
40
60
80
POD 1
POD 2
POD 3
POD 4
POD 5
POD 6
POD 7
I-13
I-17
I-21
I-23
I-27
I-28
I-29
I-30
I-32
I-34
I-36
I-40
I-41
I-42

Figure 7.


A.
DPM OF TRITIATED THYMIDINE x10⁻³
150
125
100
75
50
25
0
SF
10%
1
2
4
5
7
DAY AFTER SURGERY
B.
PERCENT OF CONTROL (SERUM FREE)
250
150
50
-50
-150
10%
IV-8
I-1
I-2
I-3
I-4
I-5
I-6
I-7
I-8
I-9
I-12
I-13
I-15
I-17